Current Topics in Genome Analysis

Cytogenetic Methods

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Introduction

Cytogenetic methods for genome analysis offer a variety of applications. One of the first application was the analysis of metaphase chromosome by banding techniques, termed karyotyping. Karyotyping based on G-banding has been of tremendous value in the past to identify chromosomal aberrations associated with inherited and acquired constitutional syndromes and birth defects, and for the description of recurring chromosomal aberrations in cancer cells. The latter has provided ample entry points for the positional cloning of cancer causing and promoting genes. One important advantage of karyotyping is the possibility to search an entire genome for chromosomal aberrations in a single experiment. There are several textbooks and lab manuals that summarize cytogenetic techniques, a comprehensive one being: "The AGT Cytogenetics Laboratory Manual" MJ Barch, T Knutsen, JL Spurbeck (Eds.), Lippincott-Raven (1997). However, the limited resolution and the often very complex nature of cytogenetic changes in cancer cells prompted the development of alternative and complementary methods for the cytogenetic evaluation of genomes. All of these recently developed methods rely on fluorescence in situ hybridization (FISH) with labeled DNA probes, hence the introduction of the term “molecular cytogenetics”. FISH can be used to map anonymous DNA clones to metaphase chromosomes, a simple and fast procedure. Initiatives to saturate the genome

with high resolution mapped, STS-tagged BAC-clones are underway (see the chapter on the Cancer Chromosome Aberration Project, Ccap, in this handout), and provide elegant tools to verify suspected chromosomal aberrations and to place these changes on the emerging sequence map of the human genome (see Kirsch et al., 2000, and the website http://www.ncbi.nlm.nih.gov/CCAP/). FISH onto extended chromatin preparations, such as DNA-Halos or mechanically stretched DNA fibers can be used to deduce the orientation of individual clones in a contig.

FISH also forms the basis for recently developed methods for the screening of cancer genomes for recurring chromosomal abnormalities have been devised: comparative genonic hybridization (CGH) has become an indispensable tool in cancer cytogenetics and is described in detail in this handout (for review see Forozan et al., 1997 and Ried et al., 1997). More recently, CGH has also been possible in a DNA-chip format (Solinas-Toldos et al., 1997; Pinkel et al., 1998). Spectral Karyotyping (SKY) and m-FISH are procedures for the simultaneous visualization of all chromosomes (of human and mice) in specific colors (Schröck et al., 1996; Liyanage et al., 1996). These techniques have become increasingly valuable for the identification of chromosomal aberrations in cells from leukemias and lymphomas, in solid tumors (and their respective animal models), and for the characterization of marker chromosome of unknown origin in prenatal and postnatal diagnostics. SKY is described on page XXXX of this handout.

Finally, FISH-based techniques are the basis for many investigations that are aimed at elucidating the 3-dimensional architecture of the interphase nucleus, and to correlate chromatin structure with gene function (for a review see Cremer et al. 1993 and Zink and Cremer, 1998).

Fluorescence in situ hybridization (FISH)

DNA in situ hybridization is a technique that allows the visualization of defined sequences of nucleic acids at the cellular and subcellular level. The method is based on the site specific annealing (hybridization) of single-stranded DNA molecules (probes) to denatured, complementary sequences (targets) on cytological preparations, like metaphase chromosomes or interphase nuclei. After fluorescence detection steps, the probe sequences become visible at the site of hybridization.

FISH is a multistep procedure. Specific protocols for each of the steps are provided in Part II. The single steps are, simplified, summarized as follows.

1. Preparation of DNA probes
2. Labeling of DNA probes
3. Preparation of cytological specimens
4. Denaturation of probe and specimen
5. In situ hybridization
6. Fluorescence probe detection
7. Fluorescence microscopy

1. DNA preparation usually follows standard procedures. Modifications are described in detail in Part II.

2. The labeling of DNA or RNA probes for FISH is generally performed enzymatically. Even though chemical procedures are available, the enzymatic protocols using Nick-translation, random priming, PCR or tailing with terminal transferase proved to be the simplest and most reliable labeling protocols. During the labeling reaction modified nucleotide analogs are incorporated. They are linked to haptens, e.g., biotin, digoxigenin, or Dinitrophenol. Recently, nucleotide analogs became available, that are directly conjugated to fluorochromes (Wiegant et al., 1991), such as FITC-dUTP or TRITC-dUTP.

3. The preparation of metaphase or prophase chromosomes for in situ hybridization follows standard cytogenetic protocols (Verma and Babu, 1989). FISH can be easily combined with chromosome banding protocols (Arnold et al., 1992). Interphase nuclei, e.g., amniotic fluid cells, fibroblasts, or nuclei in tissue sections need various pretreatment steps in order to increase probe accessibility and to reduce fluorescence background staining.

4. The probe molecules and the target DNA are denatured thermally. Formamide is added to reduce the melting temperature of the double stranded DNA. If complex DNA probes are used, an additional preannealing step with an excess of unlabeled total genomic DNA or the Cot1-fraction of human DNA prior to the hybridization is required, leading to the term chromosomal in situ suppression (CISS) hybridization (Cremer et al., 1988).

5. The hybridization reaction is usually carried out at 37°C for about 16 hours. Shorter hybridization times (minutes to hours) are sufficient for probes that detect repetitive sequence motifs. Certain probes require increased hybridization temperatures in order to exclusively label their target region. If entire genomes are hybridized, e.g. using comparative genomic hybridization (CGH), prolonged hybridization times are necessary.

6. The detection reaction is performed indirectly with fluorochromes linked to avidin or antibodies against the reporter molecules. If probes are used that are conjugated with fluorochromes, detection steps are not required. Numerous fluorochromes are available including fluorochromes emitting in the blue (AMCA, Cascade blue), in the green (FITC), and in the red (TRITC, Texas red, rhodamine, Cy3). More recently, fluorochromes which emit in the infrared, such as Ultralite 680 or Cy5, became commercially available.

7. Probe signals are visualized by epifluorescence microscopy. New generations of specific filter sets allow to precisely separate the fluorochromes (Ploem and Tanke, 1987; Marcus, 1988). A convenient development, in particular with respect to the needs of routine diagnostic laboratories provide double or triple band pass filters (Johnson et al., 1991). They are used to simultaneously visualize several fluorochromes. Digital imaging devices with a high photon detection efficiency and a high dynamic range, like silicon intensified tube cameras or charge coupled device (CCD) cameras add significantly to the sensitivity and provide the basis to quantify fluorescence images (Hiraoka et al., 1987). CCD cameras are also sensitive over a broad spectral range, thus fluorochromes emitting in the infrared spectrum can be included as fluorescence detection systems. Confocal laser scanning microscopy is preferred if light optical sectioning of three dimensional specimens, like interphase nuclei, is desired (Cremer and Cremer, 1978).

Complementing each other, these developments have contributed to the tremendous improvements of FISH over the last few years with respect to sensitivity, resolution, and multiplicity. DNA or cDNA probes as small as 500 bp can be visualized on metaphase chromosomes. This equals the sensitivity of isotopic detection formats. The spatial resolution of fluorescence signals is higher than the one involving radioisotopes

and is, on metaphase chromosomes in the range of some 5 Mbp. However, the less condensed interphase chromatin increases the resolution to approximately 100 kbp (Lawrence et al., 1990; Trask et al., 1991). Recently, several techniques for extended chromatin preparations have improved the resolution power dramatically (Heng et al., 1992; Wiegant et al., 1992; Parra and Windle, 1993). Using histone depleted interphase nuclei and other high resolution FISH techniques, the spatial resolution is in the range of 5 kbp (Tocharoentanaphol et al., 1994). The improvements of sensitivity and spatial resolution have had considerable impact on gene mapping and studies dealing with the 3D organization of chromatin in interphase nuclei (Florijn et al., 1995; Heiskanen et al., 1996; Michalet et al., 1997).

The possibility of visualizing several chromosomal targets simultaneously has broadened the spectrum of diagnostic and research applications of FISH and has become one of the most attractive features of FISH analysis. The number of suitable labeling and detection formats still limits the multiplicity of FISH. To overcome these limitations, approaches using combinatorial or ratio labeling of single probes have been devised. They increase the number of target regions that can be discerned by means of their respective color after a single hybridization experiment beyond the number of available fluorochromes (Nederlof et al., 1990; Ried et al., 1992a,b; Dauwerse et al., 1992; Wiegant et al., 1993; Lengauer et al., 1993). For example, with three labeling and detection systems, a total of seven probes can be distinguished. Probes 1, 2, and 3 would be visualized as a pure fluorochrome, while probes 4-7 would appear as fluorochrome mixtures as follows: probe 4, FITC and TRITC; probe 5, FITC and AMCA; probe 6, TRITC and AMCA; and probe 7, FITC, TRITC, and AMCA. Using digital image analysis fluorochromes emitting in the infrared can be included. Thus, DAPI might be used to counterstain the chromosomes which gives additional information. The

possibility to use digital imaging devices for an accurate quantification of FISH signals also forms the basis for the newly introduced technique of comparative genomic hybridization (CGH).

An equally important improvement for the application of FISH in medical diagnosis is the availability of different probe sets. This increases the flexibility to design experimental protocols to specifically address the diagnostic requirements. One of the first probes used for FISH analysis comprised cloned DNA fragments that contained consensus sequences for the repeat units of centromeric or paracentromeric heterochromatin of specific chromosomes. Using appropriate stringency conditions the centromeric region of almost every human chromosome can be visualized specifically (Willard and Waye, 1987; Vogt, 1990). The limitations of these probes, however, are obvious. Since all chromosome specific repetitive DNA reported to date is localized to discrete subregions of each chromosome, this class of DNA probes is unsuitable for the analysis of many types of chromosomal aberrations, e.g. translocations and deletions involving chromosomal arms. Their use is, therefore, with few exceptions restricted to the assessment of numerical aberrations.

These limitations were overcome with the advent of composite probe sets specific for entire chromosomes, also termed chromosome painting probes (Pinkel et al., 1988; Cremer et al., 1988; Lichter et al., 1988). Based on the enrichment of individual chromosomes by means of flow cytometry, fragments of isolated chromosomes were cloned in phage and plasmid vectors (Collins et al., 1991). Since genomic DNA clones do not only contain chromosome specific single copy sequences, but also highly repetitive elements of the SINE and LINE families (for review see, e.g., Vogt, 1990), the successful delineation of individual chromosomes depends on the use of suppression hybridization protocols (Cremer et al., 1988). An excess of unlabeled DNA derived from the Cot1-

fraction of human DNA is used to block the cross hybridization of ubiquitously distributed repetitive DNA fragments.

The rapid progress of DNA cloning technology and the success of the Human Genome Project made an increasing number of region or gene specific DNA clones available that can be used to pinpoint specifically the variety of chromosomal aberrations involved in human genetic diseases. In complementation to these developments, efficient protocols became available in order to selectively enrich the human DNA content in a background of e.g. hamster (Lengauer et al., 1990) or yeast DNA (Nelson et al., 1989; Lengauer et al., 1992a).

Rather recently developed probe generations include region specific probes derived from microdissected human chromosomes (Lengauer et al., 1991; Meltzer et al., 1992, Guan et al., 1994) and probes generated from cytogenetically detected marker chromosomes, a procedure termed "reverse painting" (Carter et al., 1992). Finally, entire genomes can be used as probes (Kallioniemi et al., 1992; Kallioniemi et al., 1993; du Manoir et al., 1993; Speicher et al., 1993, Schröck et al., 1994, Ried et al., 1994). In a comparative hybridization format, these probes are used to reveal partial or complete chromosome gains and losses in test genomes, e.g., in DNA extracted from solid tumor cells.

Since the first report on in situ hybridization protocols by Gall and Pardue (1969), FISH has evolved as a powerful and versatile experimental tool in genetic research. In basic research, FISH contributed to the understanding of nuclear topography, both of mammalian and plant cells. The experimental evidence, established after UV-laser microirradiation of interphase nuclei, that chromosomes are organized as discrete territories in the cell nucleus (Cremer et al., 1982a,b) was confirmed elegantly using DNA from hybrid cell lines (Manuelidis, 1985; Schardin et al., 1985) or cloned DNA

libraries from individual chromosomes as probes (Cremer et al., 1988; Lichter et al., 1988a; Pinkel et al., 1988). The distribution of chromosome centromeres was investigated by FISH in interphase cells of tissue sections and isolated nuclei, and provided evidence for a non-random, cell type specific arrangement (Manuelidis, 1984; Arnoldus et al., 1989; Haaf and Schmid, 1989; van Dekken et al., 1990; Popp et al., 1990; Arnoldus et al., 1991; Weimer et al., 1992). Furthermore, taking advantage of confocal laser scanning microscopy, subchromosomal compartments were defined and the distribution of certain genes with respect to the chromosome domain was successfully investigated (Spector, 1990; Spector et al., 1991; Zirbel et al., 1993; Cremer et al., 1994). By means of RNA in situ hybridization, a distinct compartmentalization of transcriptional mechanisms was determined (Lawrence et al., 1989; Carter et al., 1991; Carter et al., 1993; Xing et al., 1993).

Fluorescence in situ hybridization to metaphase chromosome preparations revealed distinct structural features of the arrangement of repetitive DNA sequences, as well as the nonrandom distribution of genes with respect to chromosome bands (Korenberg and Rykowski, 1988; Chen and Manuelidis, 1989; Holmquist, 1992). Chromatin packaging models were assayed by FISH with DNA clones for specific genes and revealed a fixed lateral position on metaphase chromosomes (Baumgartner et al., 1991). Selig et al. (1992) used FISH to monitor replication timing in a series of different cell types, and mapped the replication timing topography of the cystic fibrosis locus. Another study revealed an allele-specific replication timing (Kitsberg et al., 1993).

FISH to meiotic chromosomes of human and mouse origin was used to track down basic events in meiosis, such as nondisjunction and recombination (Pieters et al., 1990; Guttenbach and Schmid, 1991; Ashley et al., 1994).

Comparative mapping studies with human DNA probes to chromosomes from great apes, hylobatids, old world monkeys and prosimians, established a molecular taxonomy. As yet unidentified chromosomal rearrangements that occured during the course of mammalian chromosome evolution were delineated with high resolution. Thus, entire karyotypes were reconstructed with chromosome specific DNA libraries and region specific DNA probes (Wienberg et al., 1990; Wienberg et al., 1992; Jauch et al., 1992; Ried et al., 1993a).

Based on suppression hybridization with cosmid and YAC clones (Landegent et al., 1987), FISH was introduced as an important method to the international efforts of the human genome project. Large numbers of DNA clones could be mapped on human metaphase and prometaphase chromosomes by means of fractional-length measurements (Lichter et al., 1990a) or, combined with cytogenetic banding techniques (Klever et al., 1991; Baldini and Ward, 1991; Arnold et al., 1992) with respect to chromosome bands (Ward et al., 1991; Bellanné-Chantelot et al., 1992; Cohen et al., 1993). The hybridization of low complexity cDNA clones made it also possible to rapidly assess the chromosomal location of candidate disease genes, and to compare these mapping positions with data from, e.g., genetic linkage studies based on pedigree analysis (Ried et al., 1993c).

The application of FISH to problems in medical diagnosis are numerous (for a review see, e.g., Tkachuk et al., 1991): in clinical cytogenetics, FISH analysis is often a helpful adjunct to chromosome banding studies, and is used to confirm, or in some cases even to allow to determine the origin of marker chromosomes and to highlight numerical and structural aberrations (Jauch et al., 1990; Popp et al., 1993). In general, FISH has the distinct advantage that the diagnosis of numerical and structural chromosomal aberrations is not restricted to dividing cells, i.e., metaphase chromosomes, but is applicable during

all stages of the cell cycle, a feature termed interphase cytogenetics (Cremer et al., 1986). Interphase cytogenetics has become an useful diagnostic tool in cancer cytogenetics (Nederlof et al., 1989; Tkachuk et al., 1990; Ried et al., 1992c; Lengauer et al., 1992a; Ried et al., 1993b). Also, the diagnosis of trisomy 21 is performed in many laboratories on a routine basis directly in interphase nuclei (Klinger et al., 1992; Klever et al., 1992; Ward et al., 1993). The high resolution of FISH analysis allows for a sensitive visualization of even submicroscopical deletions. This has implications for the diagnosis of constitutional microdeletion syndromes (Ledbetter, 1992a), the diagnosis of carrier status in X-chromosomal recessively inherited diseases associated with deletions (Ried et al., 1990), and the identification of deletions of tumor suppressor genes in certain types of human malignancies (Stilgenbauer et al., 1993). Pathogen based diagnostic procedures, such as the detection of virus genomes in tissue sections have been reported (Brigati et al., 1983). The development of biological dosimeters for a follow up and long term screening of individuals who were exposed to radiation, is an useful tool to determine the effects of ionizing substances, resulting in dicentric chromosomes and translocations (Cremer et al., 1990; Popp and Cremer, 1992; Gray et al., 1992).

Comparative genomic hybridization (CGH)

Comparative genomic hybridization is a new molecular cytogenetic technique based on quantitative two color fluorescence in situ hybridization (Kallioniemi et al., 1992; Kallioniemi et al., 1993; du Manoir et al., 1993). CGH allows, in a single experiment, to detect genetic imbalances in solid tumors or any desired test genome, and to determine the

chromosomal map position of gains and losses of chromosomes or chromosomal subregions on normal reference metaphase preparations. Total genomic DNA from a tumor specimen is isolated following standard procedures. A reference, or control DNA is isolated from an individual with a normal karyotype (46,XX or 46,XY). DNA extracted from non-involved tissue of a tumor patient can be used as well as reference DNA. The two genomes are labeled differentially with reporter molecules (e.g., biotin-dUTP for the tumor genome, and digoxigenin-dUTP for the reference genome) in a standard nick translation reaction. The so labeled genomes are then pooled and hybridized to reference human metaphase spreads (46,XY). In order to reduce the cross hybridization of highly repetitive sequences present in both genomes, an excess of unlabeled Cot1-fraction of human DNA is included in the hybridization mixture. This step is necessary because the high hybridization kinetics of repetitive DNA might impair the evaluation of the single copy sequences that are over- or underrepresented in the tumor genome. In a subsequent step, the hybridized probes (genomes) are visualized with different fluorochromes (e.g. avidin-FITC, green fluorescence, for the biotinylated tumor genome and anti-digoxigenin coupled to rhodamine, red fluorescence, for the reference genome). The differences in fluorescence intensities along the chromosomes on the reference metaphase spread reflect the copy number of corresponding sequences in the tumor DNA. If chromosomes or chromosomal subregions are present in identical copy numbers in both, the reference and the tumor genome, the observed fluorescence is a blend of an equal contribution of red and green fluorescence. If chromosomes are lost or chromosomal subregions deleted in the tumor genome, the resulting color is shifted to red. A gain of a certain chromosome in the tumor would be reflected by a more intense green staining on the respective chromosome in the reference metaphase preparation. Increased supernumerary, e.g. local DNA amplification results in green signals of similar shape and intensity as single copy

probes, e.g., YAC clones. In many instances, gross chromosomal aberrations in tumor genomes, such as high level DNA amplifications, are visible directly in the fluorescence microscope. However, a quantitative measurement of fluorescence intensity values based on digital image analysis is crucial for a precise CGH analysis of low copy number changes. This analysis includes image acquisition of the rhodamine and FITC fluorescence with a CCD camera. Using custom computer software, the painted chromosomes are then segmentated and the fluorescence values determined perpendicular to the axis of the chromosome on a pixel to pixel basis. The result of the measurement of the fluorescence values can now be visualized by means of a look up table where certain colors refer to gains or losses in the tumor genome. The final step in a quantitative fluorescence measurement includes the calculation of average ratio profiles along the chromosomal axis based on data from at least 5 metaphase spreads. Values of 1 indicate equal copy numbers of the respective chromosomes in the tumor and test genome, a ratio of 0.5 a deletion of one homologous chromosome and ratios of 1.5 reflect a trisomy in the tumor. Gene amplifications can be mapped to reference metaphase chromosomes according to peak fluorescence ratios of more than 2.5. For a detailed description of the CGH-software the reader is referred to du Manoir et al., (1995).

The validity of CGH to delineate complex genetic changes in solid tumors has been investigated in several studies. Using a cell line established from a renal cell carcinoma, the results from karyotype analysis were compared with CGH. All chromosomal aberrations detected after karyotyping could be confirmed after the CGH analysis (Du Manoir et al., 1993). Another, independent study to verify the results of CGH analysis was described by Schröck et al. (1994) with a series of human gliomas. In this sample collection, banding was often impossible due to inferior spreading of the metaphase chromosomes and the frequent observation of DM chromosomes. By means of

interphase cytogenetics with YAC clones for chromosomal subregions that revealed gains and losses after CGH, the presence of all imbalances could be confirmed in interphase nuclei prepared from tissue sections, i.e., ratios of 1.5 after CGH were in accordance with three signals in interphase nuclei. In addition, a DNA amplification that was mapped to chromosome 4 by CGH, was shown to be present in DM chromosomes of this tumor after FISH with a chromosome 4 specific DNA library to metaphase chromosome preparations. Also, the amplification of the EGFR-gene determined by DNA fingerprint analysis resulted in peak fluorescence values on chromosomal map position 7p12, known to harbor the gene encoding this growth factor receptor.

One of the distinct advantages of CGH is the fact that tumor DNA is the only requirement for this molecular cytogenetic analysis. Thus, archived, formalin fixed and paraffin embedded tissue can be used as well (Speicher et al., 1993). This allows to establish a correlation of the microscopic phenotype and the genotype in solid tumors (Speicher et al., 1995). In combination with microdissection of distinct areas on microscopically defined tissue sections, CGH offers a new experimental approach to study chromosomal aberrations that occur during solid tumor progression (e.g., Ried et al., 1995; Schröck et al., 1996; Ried et al., 1996; Heselmeyer et al., 1996).

CGH-technique and applications have been reviewed in the following manuscripts:

Forozan et al., (1997); Ried et al., (1997); Knuutila et al., 1998).

Spectral karyotyping (SKY)

With all the advantages and the particular elegance of comparative genomic hybridization one should not overlook its limitations. CGH allows one to identify only those chromosomal aberrations that result in DNA copy number changes. For instance, a chromosomal aberration such as the Philadelphia chromosome -arguably an important event in the transformation of hematological cells of myeloid origin, would remain undetected by CGH. Also, the chromosomal mechanisms by which individual cells generate copy number changes, e.g., duplications, isochromosome formations, dmins, hsr's, and others, remain elusive. And lastly, at the present stage of technology development, CGH generates an average of the most common aberrations in tumor genomes, disregarding important features such as clonal heterogeneity, which provides tumors with the genetic diversity to react more flexibly to environmental changes. FISH, using the plethora of available probe sets is an important technique to analyze chromosomal aberrations on a single cell level. However, a targeted analysis of, e.g., the deletion status of a tumor suppressor gene, leaves the rest of the genome unanalyzed. Therefore, the cytogeneticist would like to add to the methodological spectrum an approach that allows to visualize all human chromosomes in different colors. The goal to increase the number of chromosomal targets that can be discerned simultaneously, i.e., the multiplicity of FISH experiments has long been perceived (Nederlof et al., 1990; Ried et al., 1992). The scarcity of suitable probe labeling and fluorescence detection formats, however, makes this a non-trivial task. This is mainly due to the nature of the fluorochromes itself. In many instances, the emission spectra of fluorochromes overlap. Therefore it is difficult to discern an ever increasing number of fluorochromes using conventional, fluorochrome specific optical filters, and color karyotyping was not

possible until recently when Speicher and colleagues reported the FISH-based discernment of all human chromosome using sequential exposures with 6 different optical filters (Speicher et al., 1996). We have developed a novel approach for the visualization of FISH experiments. In strong contrast to conventional epifluorescence filter technology, we have explored the possibility of using spectral imaging to distinguish multiple and overlapping fluorochromes simultaneously, and hence achieved the goal of color karyotyping human (and other species) chromosomes (Schröck et al., 1996; Liyanage et al., 1996).

Spectral imaging refers to a novel imaging technique for the analysis of FISH experiments (Schröck et al., 1996). The application to karyotype analysis is termed spectral karyotyping (SKY) and is based on spectral imaging (Malik et al., 1996; Garini et al., 1996).

The application of spectral imaging to the field of cytogenetic research and diagnostics is termed spectral karyotyping, or SKY. In the following we will describe some of these applications to chromosome analysis both in human malignancies as well as in animal models of certain tumors, and we will try to paint a picture on how cytogenetic diagnostics might be performed in the near future.

Spectral karyotyping of human chromosomes is based on the simultaneous hybridization of a 24 chromosome specific probe pool. Chromosome specific probe pools, or chromosome painting probes, can be generated from flow-sorted human chromosomes (Telenius et al., 1992) or by chromosome microdissection (Guan et al., 1994). In order to produce a chromosome specific spectrum after hybridization, each chromosome library

was labeled either with a single fluorochrome or with specific combinations of two or three fluorochromes, allowing us to increase the number of discernible targets beyond the number of fluorochromes that are suitable for DNA-labeling. Using combinatorial labeling with five different fluorochromes, 31 different targets can be distinguished. The hybridization was visualized using spectral imaging through a single optical filter that allowed for the excitation of all fluorochromes, and the measurement of their emission spectra without the need to change from one fluorochrome specific optical filter to another. The applications of SKY to visualize chromosomal aberrations involved in human diseases are manifold. Chromosome banding based karyotype analysis is routinely performed in the prenatal and postnatal cytogenetic laboratory. The benefits of SKY in this field include (i) the identification of subtle chromosomal aberrations such as the translocation of telomeric chromatin that is difficult to detect using banding alone and (ii) the identification of small markers that remain elusive after banding. In a recently conducted study of cases with unidentified constitutional chromosome abnormalities SKY was able to refine karyotype interpretation in the majority of the cases (Schröck et al., 1997). SKY, in combination with chromosome banding analysis might also enable the automation of karyotype analysis in the clinical cytogenetic laboratory where the majority of the karyotypes are actually normal. However, the need to complement karyotype analysis with SKY is even more obvious in tumor cytogenetics. This is due to certain, characteristic features of metaphase chromosomes from malignant cells. In many instances the mitotic index is low. As a consequence, the few cells that are available would preferably be analyzed as comprehensively as possible. Also, tumor metaphase preparations, in particular those established from solid tumors and lymphomas are often of poor quality which precludes high resolution banding analysis. The matter becomes even more complicated because tumor karyotypes are often highly rearranged. This

shuffling of chromosomal segments makes it extremely difficult to identify the origin of translocated chromatin because the sequence of chromosomal bands is obscured. This problem could be overcome by adding color information that unambiguously identifies the origin of rearranged chromosomal material. Indeed, it has been shown that the combination of banding and SKY allows one to identify marker chromosomes and also chromosomal breakpoints with higher accuracy than in the past (Veldman et al., 1997). Lastly, SKY was successfully used to characterize chromosomal structures such as dmin's, hsr's, and other cytogenetic reflections of oncogene amplification whose origin could not be deduced by banding methods (Macville et al., 1999; Sawyer et al., 1998).

Systematic integration of cytogenetic data with genome maps and available probes: the Cancer Chromosome Aberration Project (Ccap)

(http://www.ncbi.nlm.nih.gov/CCAP/)

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Summary

The National Cancer Institute, USA, has established an initiative, the Cancer Chromosome Aberration Project (Ccap), to link and integrate the physical and genetic maps of the human genome with cytogenetic maps and maps of chromosomal rearrangements in human diseases. This will be achieved by high-resolution FISH mapping of colony-purified BAC clones spaced at 1-2 Mb intervals across the entire genome. All BAC clones will be anchored on the physical map by the presence of a mapped STS or by sequencing of the clone itself. The generation of a publicly accessible clone repository will allow convenient distribution of these BACs. Ccap data can be correlated with other cancer-associated and genomic databases, such as the catalogue of chromosomal aberrations in cancer and the emerging full genomic sequence. We anticipate that the use of Ccap clones will expedite and refine the mapping of chromosomal breakpoints. The eventual set of approximately 3000 Ccap BACs should facilitate the production of BAC containing DNA chips for assessing copy number of genomic segments by matrix comparative genomic hybridization. In addition, the repository will provide genome-wide tools for defining chromosomal aberrations in

cytological specimens by interphase cytogenetics. The Ccap Web site illustrates the goals and progress of this initiative (http://www.ncbi.nlm.nih.gov/CCAP/).

Introduction

Since the development of chromosome banding techniques by Caspersson, Zech and Seabright (2, 19) and their subsequent application to the analysis of chromosomal aberrations in cancers, more than 1,800 recurring chromosomal breakpoints have been identified (14). Recurring chromosomal breakpoints and regions of non-random copy number changes typically point to the location of genes involved in cancer initiation and progression.

With the introduction of molecular cytogenetic methodologies based on fluorescence in situ hybridization (FISH), namely comparative genomic hybridization (9) and spectral karyotyping (SKY) (17) or m-FISH (21), even the highly "shuffled" karyotypes in carcinomas become susceptible to analysis. As a result, numerous novel chromosomal translocations and regions of recurring copy-number imbalances are now being identified (for sample reviews, see references (6, 16)). However, all cytogenetic screening techniques are limited by their spatial resolution. In cancer cytogenetics, this resolution is usually about 10 Mb, thereby leaving a considerable gap between the cytogenetic methods used for defining chromosomal aberrations and the molecular techniques used for positional cloning of genes at chromosomal breakpoints or those mapping to regions subject to genomic copy number changes. The availability of redundant, large-insert genomic libraries (e.g., BACs) provides valuable tools for identifying cancer genes. However, a major impediment to the application of genomic

technology for cancer gene identification is the lack of integration of the cytogenetic maps of cancer breakpoints with the physical maps of the human genome. Consequently, the wealth of data on recurring chromosomal aberrations in cancer cannot be seamlessly utilized for the expeditious cloning of recurring breakpoints or genes within regions of genomic imbalances.

The Cancer Chromosome Aberration Project (Ccap) aims to systematically integrate the cytogenetic and physical maps of the human genome. The high-resolution cytogenetic localization of physically mapped BACs should empower current and future investigations. In addition, the integration of these data with databases of cancer-associated recurring chromosomal breakpoints and copy number changes enhances the usefulness of existing data sets (10).

Ccap BAC collection for Human Chromosomes

The Ccap project is a work in progress, which is anticipated to yield a standard set of approximately 3000 high-resolution FISH-mapped clones by mid 2001. In the earliest days of the Ccap project the availability of reliable physical maps with established extensive BAC tiling patterns was a rate-limiting step. Therefore the first two chromosomes to which clones were mapped were chromosomes 7 and 22 where the physical mapping data were relatively far advanced. Based on the established physical maps of human chromosomes 7 (1) and 22 (4), BACs at ~1-2 Mb intervals were identified, colony purified, and subjected to high-resolution dual-color FISH mapping onto metaphase and prometaphase chromosomes. Metaphase and prometaphase chromosomes were prepared according to standard protocols. Briefly, peripheral blood lymphocytes were cultured in RPMI, synchronized with BrdU, incubated in colcemid and a hypotonic solution, and fixed in methanol/acetic acid. BAC clones were labeled by

nick translation incorporating either biotin-16-dUTP or digoxigenin-11-dUTP. In situ hybridization was performed on two, presumably sequential, clones at a time with each clone differentially labeled. Images were acquired with a CCD-camera (Sensys, Photometrics, Tucson, AZ) attached to a Leica DMRXA microscope (Leica, Wetzlar, Germany), and fluorochrome specific optical filters (Chroma Technology, Inc., Brattleboro, VT), using Q-FISH image analysis software (Leica Imaging Systems, Cambridge, UK). An example of the mapping procedure is presented in Figure 1 for dual-color FISH with clones from chromosome 22. All selected clones contained at least one mapped STS.

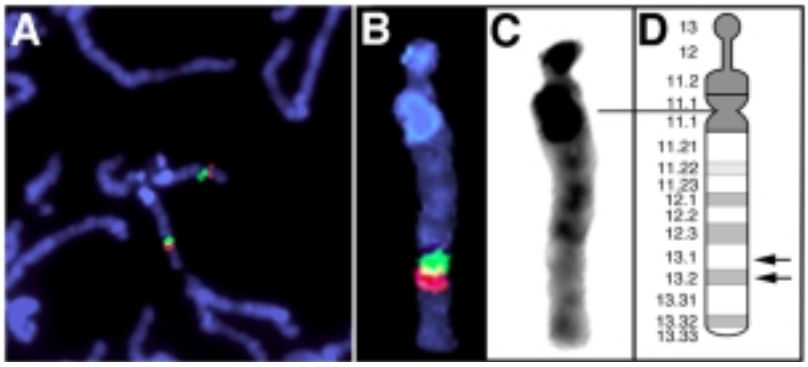

Figure 1: *Two-color high-resolution FISH mapping of chromosome 22 BACs. Metaphase chromosomes were prepared to provide a resolution of approximately 1000 bands per genome (24). FISH analysis was then performed with clone bK390B3 (green fluorescence) and bK229A8 (red fluorescence; Panel A). To facilitate band assignment, suitable chromosomes were extracted from the image (B), contrast inverted (C), and displayed next to a chromosome ideogram (D). Arrows to the right side of the ideogram indicate the corresponding map positions.*

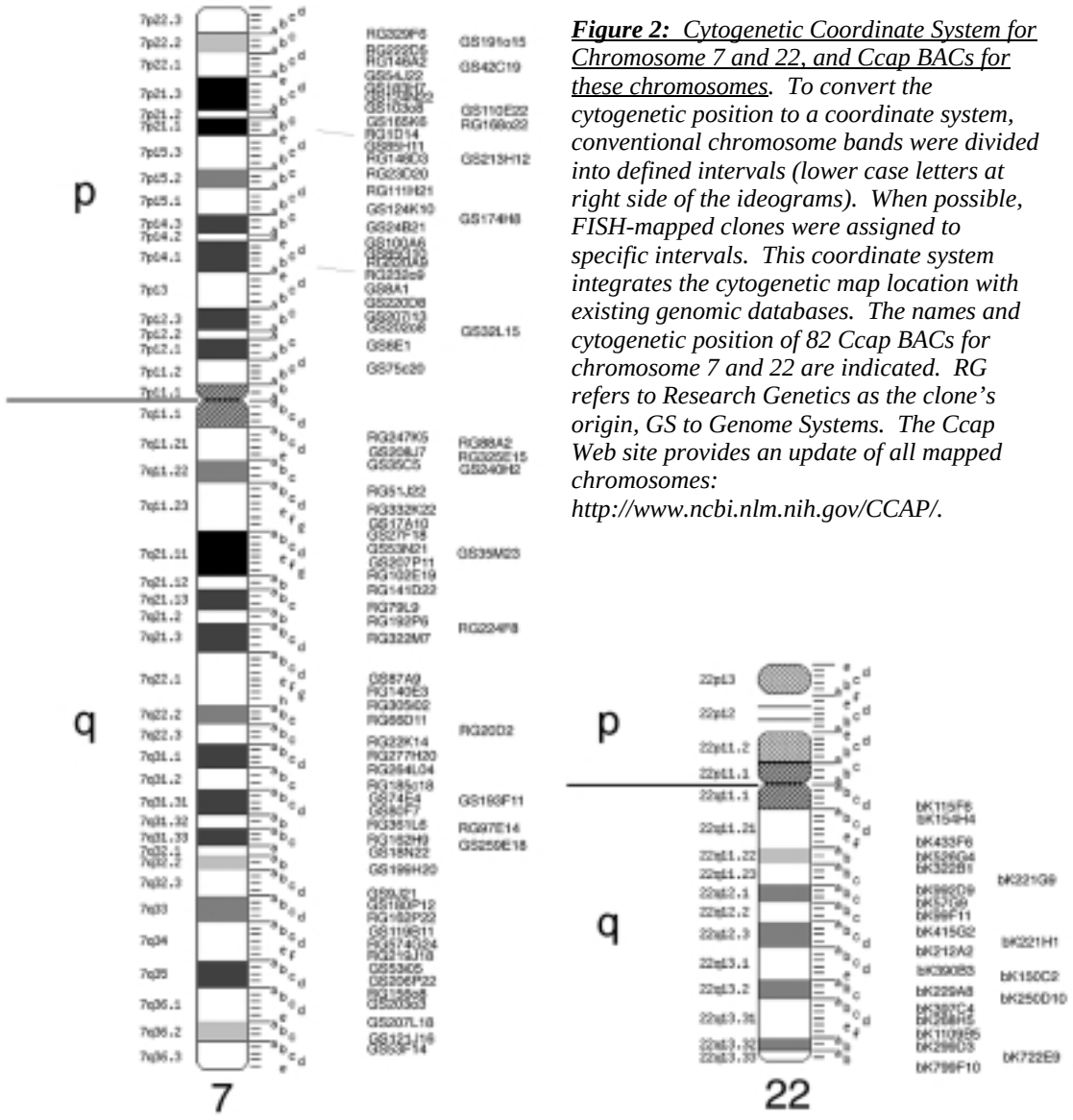

Figure 2: *Cytogenetic Coordinate System for Chromosome 7 and 22, and Ccap BACs for these chromosomes. To convert the cytogenetic position to a coordinate system, conventional chromosome bands were divided into defined intervals (lower case letters at right side of the ideograms). When possible, FISH-mapped clones were assigned to specific intervals. This coordinate system integrates the cytogenetic map location with existing genomic databases. The names and cytogenetic position of 82 Ccap BACs for chromosome 7 and 22 are indicated. RG refers to Research Genetics as the clone's origin, GS to Genome Systems. The Ccap Web site provides an update of all mapped chromosomes: http://www.ncbi.nlm.nih.gov/CCAP/.*

Based on the estimated size of chromosome 7 (170 Mb), the localization of 84 BACs provides an average spacing of ~2 Mb between clones. Using the 850 band per 7 contains 42 metaphase bands. The relative order of all adjacent BAC pairs was established unambiguously. This increases the mapping resolution to 84 positions on chromosome 7, which, when extrapolated for the entire genome, would allow for the discernment of 1700 positions. In fact, for the purposes of defining genomic coordinates that allow for sequential clone ordering within a given band where no other cytogenetic landmarks can be discerned, all bands were further arbitrarily subdivided into regularly

spaced intervals labeled with lower case letters. This subdivision into intervals was performed on the 850 band per genome ISCN ideogram (13). For chromosome 7, 160 such intervals were defined. For chromosome 22, 62 intervals were defined (Figure 2). While most of the ISCN ideogram bands were in accordance with our BrdU-banding pattern, band 7q32.2 seemed consistently closer to 7q33, with a corresponding larger 7q32.1 band. This is actually more consistent with the photographs provided in the ISCN 1995 (13). Therefore, the distance between BACs GS259E18 and GS18N22 (which mapped to 7q32.1) is actually greater than that indicated in Figure 2, which correlates with the physical map positions of these clones. For chromosome arm 7p, 1 of the 32 mapped BACs hybridized to an additional site on another chromosome. This clone was replaced with a BAC containing an adjacent STS; the new clone hybridized to a single site on 7p. Chromosome arm 7q was a bit more problematic. Ultimately, 16 of the 7q BACs were found to hybridize to more than 1 site in the genome. RG5C19, which maps within 7q11.23 showed a signal compatible with a local duplication, and an additional signal on chromosome band 7q22.2. The same problem appeared with the replacement clone that was chosen from a neighboring STS. A gap remains within chromosome band 7q22.1. The observed FISH-based mapping order for the chromosome 7 BACs was identical to that predicted from the STS-based physical map (1).

For the ~35-Mb chromosome 22, a set of 22 BACs was identified, providing a mapped clone on average every ~1.6 Mb. FISH mapping allowed precise localization of each BAC (Figure 2); additional sites of hybridization were not observed for any of the chromosome 22 BACs. The relative size and position of bands correlated well between the ideogram and the observed banding pattern.

Using additional clone sets, high-resolution FISH mapping has been completed for chromosomes 1, 5, 7, 12, 14, 16, 18, and 22 (as of 5/1/00). Additional BACs were

provided from the “BAC/PAC Resources” by Pieter de Jong and Norma Nowak, RPCI, Buffalo, NY, Vivian Cheung, U. Penn, Philadelphia, PA and R. Kucherlapati, Albert Einstein College of Medicine, Bronx, NY. The updated list of completed chromosome specific BAC sets can be viewed at the Ccap Web site (http://www.ncbi.nlm.nih.gov/CCAP/)). As work on the completion of a “rough draft” of the human genome sequence advanced, it became clear that it was more efficient to use the sequencing center(s) itself as a source of clones for mapping and inclusion in the Ccap “set”. The initiative was subsequently modified so those clones would be selected using the established tiling patterns of the sequencing centers (particularly Washington University, St. Louis as directed by Robert Waterston). Using this resource makes possible seamless placement of the clones on the finished sequence, thereby streamlining the link between clone and physical map, defining the distance from one clone in the set to the next with a resolution at the nucleotide level, and providing a single reference source for filling in the space between any two Ccap “anchoring” clones with contiguous, validated BACs each of which had been part of the sequencing queue. This resource is also critical to filling in gaps and increasing the resolution to the desired 1 Mb for those chromosomes completed in the initial phases of the project. The utility and power of this resource will certainly be aided by the related BAC development and mapping efforts of B. Trask, (U. Washington, Seattle, WA) and Ung-Jin Kim, (Caltech, Pasadena, CA) as well as by the parallel and complementary efforts of the laboratory of J. Korenberg, (UCLA, Los Angeles, CA) (11).

Database and Web Site.

The collection of mapped BAC clones described here is offered as a shared resource for the community-wide study of chromosome aberrations. Ccap BACs can be purchased through a separate repository (Specify the " NCI Ccap BAC collection" catalogue #MB11200 and include the BAC identification and chromosome number when requesting clones from Research Genetics, Huntsville, AL; http://www.resgen.com). Easy availability of the data and integration with other genomic resources are critical issues. Ordered lists of mapped BACs, their cytogenetic positions, and contained STSs are made available through the Ccap Web Site (http://www.ncbi.nlm.nih.gov/CCAP/) as part of C-GAP (http://www.ncbi.nlm.nih.gov/CGAP). It is possible to link to additional information about each clone, such as library information, clone suppliers, estimated insert sizes, and sequencing status. BACs mapped by Ccap can often be localized within contigs, either by alignment of BAC end sequences to the finished contig, or, as noted for the more recently completed chromosomes, because they are among the clones that actually contributed to the contig (8, 18). The significance of this is that sequence data and pointers to additional clones will frequently be available for fine-scale analysis of genomic regions associated with chromosome aberrations. The STSs contained in the mapped clones may also be linked to additional information such as PCR primers and amplicon sizes, together with physical and genetic positions on a variety of maps. Taken together, these links to other genomic resources substantially enhance the primary data. Indeed, data produced by Ccap strengthen links by producing a more accurate correspondence between the coordinate systems used by cytogenetics, STS maps, and the genomic sequence.

High–resolution mapping of a multiple myeloma-associated chromosomal breakpoint

The utility of Ccap BACs is vividly illustrated by the following example (Figure 3). As part of ongoing studies of chromosomal aberrations in multiple myeloma (3), a cell line (Delta 47) derived from a myeloma patient (7) was analyzed using BACs encompassing the genes encoding immunoglobulin heavy and light chain variable and constant regions. Initial studies provided evidence that the immunoglobulin lambda light chain locus was involved in a chromosomal translocation in this cell line. The *IGL* locus appeared to remain "intact" in the derivative chromosome, without separation of the centromeric variable and telomeric constant segments (data not shown). This suggests a translocation breakpoint telomeric to the constant segment.

To characterize this aberration, seven BACs were selected from the Ccap set (in order from the centromere: bK115F6 at 22q11.1, bK154H4 at 22q11.21, bK433F6 at 22q11.21, bK526G4 at 22q11.22, bK322B1 at 22q11.23, bK221G9 at 22q11.23 and bK992D9 at 22q12.1). DNA from the clones were labeled with rhodamine (red in Figure 3) and hybridized as adjacent pairs to normal interphase nuclei simultaneously with an *IGL* constant region (*IGL-C*)-containing BAC (Genome Systems, clone GS42636) labeled with FITC (green in Figure 3). This analysis demonstrated that the *IGL–C* locus is flanked by bK526G4 centromerically and bK322B1 telomerically (see Figure 2 for positioning information). These flanking BACs were then used to characterize the myeloma-associated translocation, with the aim of successively using Ccap BACs and intervening BACs from established and evolving physical maps to identify the precise position of this chromosomal breakpoint. Fortuitously, one of the initial BACs

(bK322B1) hybridizes to both derivative chromosomes and therefore contains the region of chromosome 22 associated with the aberration (see Figure 3 g,h).

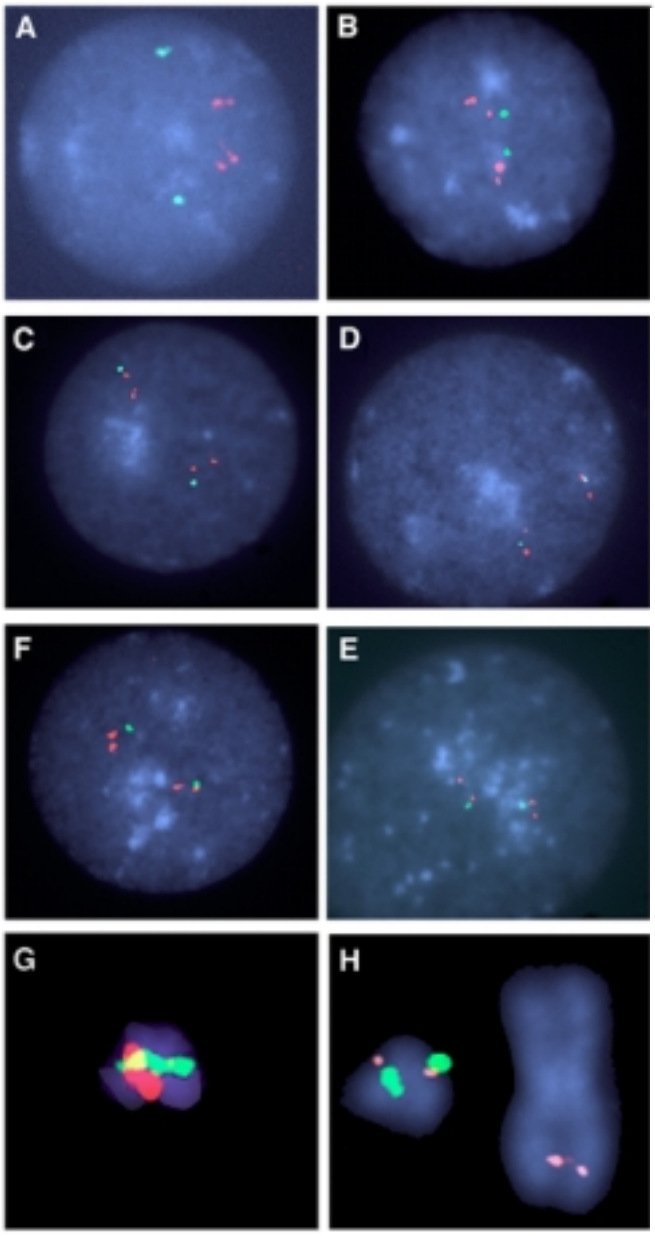

Figure 3: *Localization of a BAC containing the Igλ constant region (IGL-C) locus between two chromosome 22 Ccap clones. DNA from seven Ccap clones from chromosome 22 were labeled with rhodamine (red fluorescence) and hybridized sequentially as the following adjacent pairs to normal interphase nuclei along with a BAC containing the IGL-C locus labeled with FITC (green fluorescence): A,115F6 (22q11.1) and 154H4 (22q11.21); B, 154H4 (22q11.21) and 433F6 (22q11.21); C, 433F6 (22q11.21) and 526G4 (22q11.22); D, 526G4 (22q11.22) and 322B1 (22q11.23); E, 322B1 (22q11.23) and 221G9 (22q11.23); F, 221G9 (22q11.23) and 992D9 (22q12.1). The IGL-C locus-containing BAC can be seen to map between 526G4 and 322B1, D. G, A multiple myeloma-associated translocation splits the signal from Ccap BAC 322B1. Hybridization of a BAC containing the IGL locus labeled with FITC (green fluorescence) and clone 322B1 labeled with rhodamine (red fluorescence) to a normal metaphase chromosome 22 (counterstained with DAPI), showing signals together on the same chromosome. H, A derivative chromosome 22 and its translocation partner (from cell line Delta 47 derived from a patient with multiple myeloma) showing the IGL locus retained on the der22 and BAC 322B1 hybridization to both the der(22) (left) and the partner derivative chromosome (right); note the less intense red fluorescent signals on the two derivative chromosomes compared to the green fluorescent signal on the der(22).*

As data become available from genomic sequencing projects it will become possible to definitively locate the mapped BACs with a level of resolution that will approach and often achieve nucleotide specificity. For example, the availability of primary sequence for human chromosome 22 (5) has allowed us to locate the individual

CCAP BACs described in Figure 3 and also determine the distances between them as approximately 1195, 1120, 1420,1800, 1085, and 1745 Kb. This also provides verification of the 1-2 Mb spacing for which we were aiming.

High-resolution mapping of an acute myelogenous leukemia-associated chromosomal breakpoint

The ability of Ccap BACs to accelerate high-resolution mapping of cancer-associated chromosomal breakpoints in primary patient samples is demonstrated by the example in Figure 4. SKY analysis of metaphase chromosomes from a patient with acute myelogenous leukemia (AML) revealed a chromosomal translocation t(7;21). This aberration was undetectable by conventional cytogenetic analysis; specifically, the limited resolution and poor morphology of the chromosome preparation, a common problem in cancer cytogenetics, allowed for only a crude band assignment and a corresponding resolution < 20 Mb. The breakpoint was predicted to map within chromosomal band 7p21-22. FISH analysis demonstrated that clones GS42C19, RG146A2, RG222D5, and GS191015, all mapping within chromosome bands 7p22.1 and

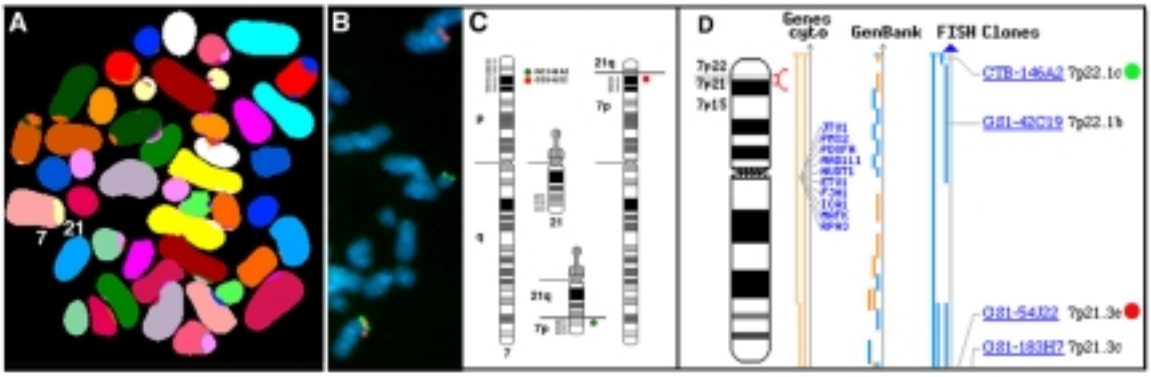

Figure 4: *Use of Ccap clones for high-resolution mapping of a chromosomal breakpoint in acute myelogenous leukemia. The chromosomal rearrangement, undetected by conventional banding analyses, was identified using SKY (A). The breakpoint on chromosome 7 was assigned to 7p21-p22. Dual-color FISH with BACs from the Ccap collection reveal the breakpoint with clones GS54J22 which remains on chromosome 7 (arrow) and GS42C19 which is translocated to chromosome 21 (arrowhead). The normal chromosome 7 is labeled with both clones (B). The schematic illustration summarizes the hybridization pattern with clones flanking the breakpoint (C). The integration of cytogenetic and sequence maps allows the placement of chromosomal translocations on the emerging sequence map. The sequence*

7p22.2, were translocated onto chromosome 21. BACs mapping within 7p21.3 (GS54J22 and GS183H7) remained on chromosome 7. These results readily provided a tremendous improvement in the resolution with which this breakpoint could be mapped. Furthermore, the availability of ordered STSs between sWSS1723 and sWSS3152 (contained within clones GS54J22 and GS42C19, respectively) should greatly assist in the identification of suitable BACs spanning the interval, one or more of which should contain the breakpoint (see http://genome.nhgri.nih.gov/chr7). In order to catalogue novel chromosomal aberrations detected by CGH and SKY, and to query these aberrations against existing cytogenetic databases we have developed a new database that, using the Ccap BAC clones, enables the seamless conversion of cytogenetic data to sequence maps.

We anticipate that this database will become available to the public towards the fall of 2000 (http://www.ncbi.nlm.gov/sky/skyweb.cgi). Figure 4D exemplifies the integration of cytogenetics and sequence information for the breakpoint on chromosome 7.

Summary and the future of Ccap

Conventional and molecular cytogenetic methods have successfully led to the identification of chromosomal aberrations in cancer cells, providing important insights about molecular genetic events that may be involved in tumor initiation and progression. For instance, the translocation t(8;14) in Burkitt's lymphoma juxtaposes the *MYC* oncogene with the immunoglobulin heavy chain gene, leading to dysregulated expression of this oncogene (22). Copy number increases within Xq12 assessed by CGH in hormone therapy refractory prostate carcinomas pointed to the amplification of the androgen receptor locus as the genetic cause for therapy resistance (23). Cytogenetically visible chromosomal deletions frequently point to the location of tumor suppressor genes: for

instance, the von-Hippel-Lindau tumor suppressor gene was suspected to map to chromosome arm 3p based on conventional chromosome banding analyses and was subsequently isolated (12). Tools that facilitate the identification of genes residing near cancer-associated cytogenetic abnormalities are critical for enhancing our understanding of cancer genetics. A persistent problem is that the resolution of available cytogenetic methods, (including CGH and SKY) is considerably lower than that needed for rapidly converting data on chromosomal rearrangements into useful entry points for gene identification. Tools that provide high-resolution linkage of the cytogenetic maps with the physical and genetic maps of the human genome are therefore needed.

The Cancer Chromosome Aberration Project (Ccap) aims to generate a repository of BAC clones with an initial resolution of ~1-2 Mb. The cytogenetic location of all clones will be established by high-resolution FISH mapping onto prometaphase chromosomes. Linkage to existing physical maps will be provided by the requisite presence of a mapped STS in all selected BACs and, in many cases, by the use of the clone in the human genome sequencing effort. Accordingly, the use of Ccap clones should allow all chromosomal breakpoints to be localized to within 2 Mb as well as provide entry points to existing physical maps of the intervening interval. With the completion of a first draft of the human genome, sequences between mapped breakpoints can than be immediately queried for potential candidate genes,

CGH has been widely applied for the detection of genomic imbalances in tumor cells. Conventional CGH analyses use normal metaphase chromosomes as targets for the mapping of copy number changes. This limits the mapping of such imbalances to the resolution limit of metaphase chromosomes (i.e., 10-20 Mb). Efforts to increase this resolution have led to the concept of matrix-CGH, whereby the chromosomal target is replaced with cloned DNA immobilized on solid supports (e.g., glass slides) (15, 20). The

resulting resolution then depends on the size of the immobilized DNA fragments. The use of clones from the Ccap repository in matrix-CGH would theoretically allow genome screening at a resolution of 1-2 Mb. The eventual use of contiguous BACs could enhance this further to 100-200 kb. Cross-reference with existing STS-based maps would also facilitate clone contig assembly and, in the long run, integration with the human sequence map. In a joint effort with the NCI intramural arraying facility, the Ccap BAC clones are being printed on glass arrays for the purpose of high-resolution matrix CGH analyses.

One critical issue is the integration of databases for the cytogenetic and physical maps. A meaningful integration would have to fulfill several requirements:

1) A direct connection between catalogued and newly discovered chromosomal breakpoints or regions of genomic imbalances with the BAC clone set.
2) Direct display of recurring chromosomal breakpoints that coincide with BAC locations.
3) Placement of mapped BAC's on the human sequence and (at least initially) tools to build contiguous clone sets.
4) The integration of the BAC clone set with databases for comparative genomic hybridization and spectral karyotyping.
5) An interface for the identification of synteny of chromosomal aberrations in human cancer and their respective mouse models.

These developments are being pursued in a collaborative effort with the National Center for Biotechnology Information (NCBI) at the National Institute of Health. A Prototype database has been developed for internal use and a publicly available version will be launched during the fall of 2000. Via Ccap clones (or other STS-mapped clones), the use of this database will allow for the seamless conversion of chromosomal breakpoints and copy alterations to the sequence of the human genome.

In summary, a systematic integration of the physical and cytogenetic maps of the human genome whose centerpiece is a set of high-resolution mapped and STS or full sequence anchored BACs is now in process.

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Protocols and Websites

The website "http://www.riedlab.nci.nih.gov" contains a comprehensive collection of updated protocols, and links to websites for supplies and reagents, mircroscope equipment, and imaging software.

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Cytogenetic Method	**Whole genome scanning**	**Resolution**	**Specific equipment/software**	**Application**
G-banding	yes	10 Mb	no	Diagnostics
CGH	yes	5-10 Mb	yes	Cancer cytogenetics
SKY	yes	2-5 Mb	yes	Diagnostics/ Cancer cytogenetics/ animal models
FISH	no	10-50 kb	no	gene mapping/break point detection
fiber-FISH	no	3-50 kb	yes	contig assembly

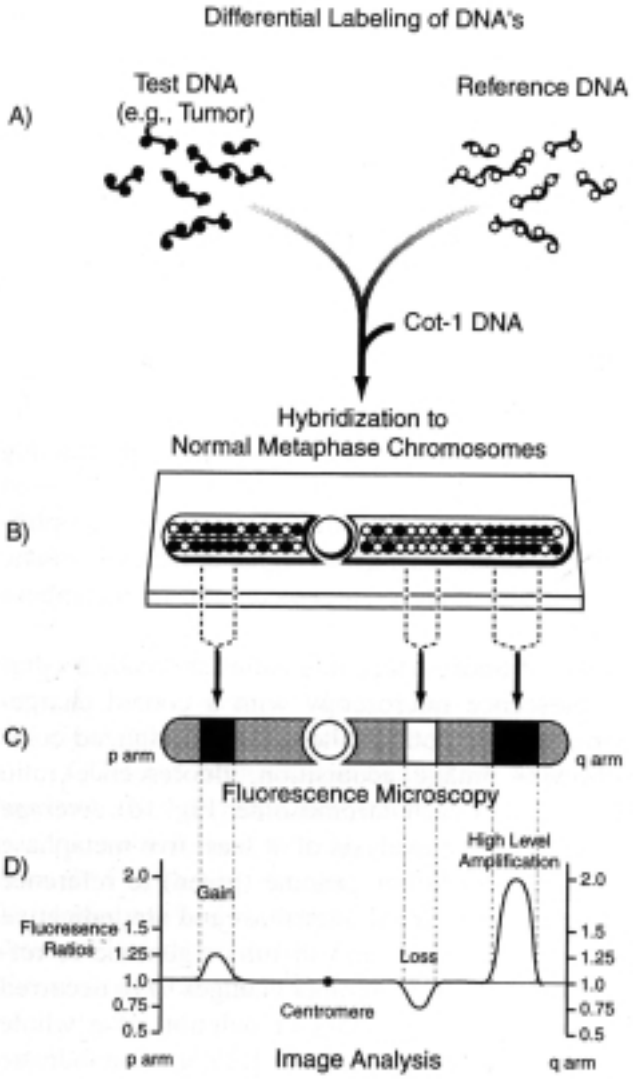

Figure 1 Schematic diagram outlining the CGH technique. (a) Test and reference DNAs are differentially labeled with fluorochromes [e.g. test DNA in green (closed circles) and reference DNA in red (open circles)] and (b) hybridized to normal metaphase chromosome spreads. (c) Fluorescence microscopy is used to generate a ratio image, which displays the differences in fluorescence intensities along the length of each chromosome, with green staining (depicted in black) indicative of tumor gain(s) and red staining (depicted in white) indicative of tumor loss(es). The areas depicted in gray represent regions of the chromosome that do not have copy number changes (''normal'' regions). (d) Fluorescence ratio profiles are calculated along the axis of each chromosome, indicative of copy number changes in the test DNA relative to the reference DNA. A ratio of 1.0 indicates that there are no copy number changes (e.g. a ''normal'' region of the chromosome), while ratios of 0.75 or less indicate a loss of DNA and ratios of 1.25 or greater indicate DNA gains at the corresponding location along the chromosome axis. Ratios of 2.0 or greater indicate the presence of a high level DNA amplification.

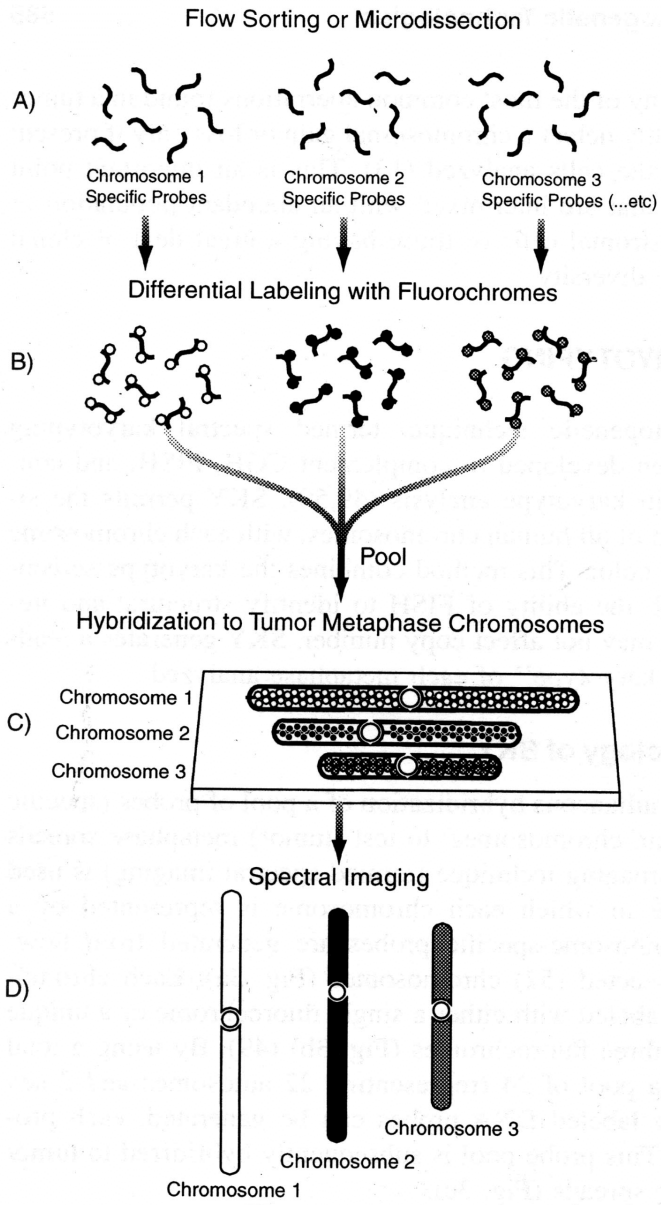

Figure 3 Schematic diagram outlining the SKY technique. (a) Chromosome-specific probes are generated by flow sorting or microdissection of chromosomes and (b) differentially labeled with fluorochromes, to produce a pool of 24 uniquely labeled probes (representing each human chromosome). (c) The probe pool is hybridized to tumor metaphase chromosomes. (d) Spectral imaging permits the simultaneous visualization of all of the chromosomes, with each chromosome "painted" in a different color. In this example, chromosome 1 is labeled with probe represented by open circles, visualized as a chromosome "painted" in white, while chromosome 2 is labeled with a probe represented by closed circles, visualized as a "black" chromosome. Likewise, chromosome 3 is labeled with a probe represented by gray circles, visualized as a chromosome "painted" in gray.